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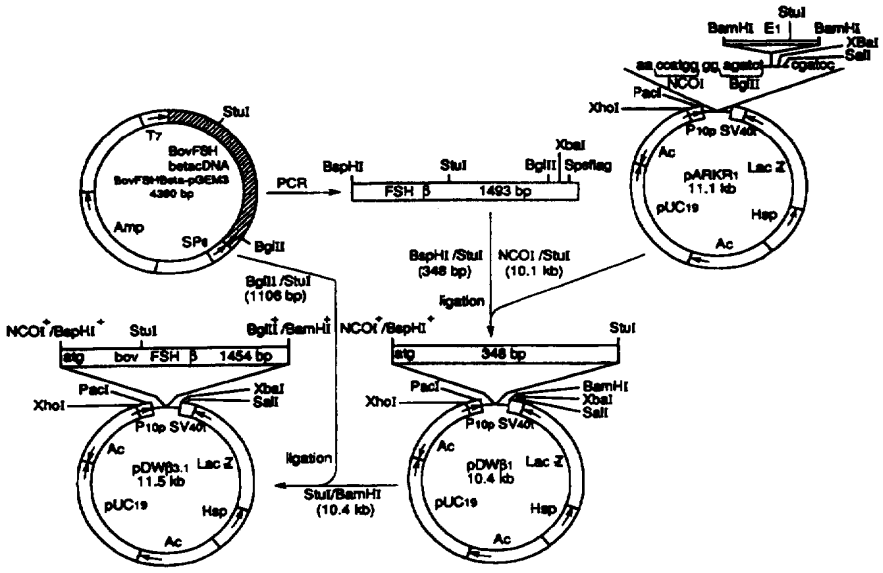
(54) Title: PRODUCTION OF BIOLOGICALLY ACTIVE RECOMBINANT BOVINE FOLLICLE STIMULATING HORMONE (REC bFSH) IN THE BACULOVIRUS EXPRESSION SYSTEM



(57) Abstract

The invention provides methods for the production of recombinant bovine Follicle Stimulating Hormone (bFSH) as well as vectors and cells for use in said methods. In particular the invention provides baculovirus based vectors which are capable of expression of bFSH in insect cells. bFSH is a heterodimeric protein belonging to a family of glycoprotein hormones which are produced in the pituitary or the placenta. It finds its use in many fertility related applications. Expression of bFSH in baculovirus/insect cell systems leads to a recombinant bFSH which has an unexpected high activity in a human FSH receptor assay and/or a bovine immature oocyte assay. The genes encoding the subunits of bFSH may be present on one baculovirus derived vector or on two or more vectors which are to be cotransfected.

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Title: Production of biologically active recombinant bovine follicle stimulating hormone (rec bFSH) in the baculovirus expression system

Introduction

This invention relates to the field of recombinant expression in insect cells. It relates especially to the expression of heterodimeric proteins in such cells and more particularly to the expression of glycoprotein hormones such as follicle stimulating hormone and the like.

Follicle stimulating hormone (FSH) belongs to the family of glycoprotein hormones, which are produced either in the pituitary (LH, TSH) or in the placenta (hCG). Within a species, each of these hormones consists of a common a subunit, which is non-covalently bound to a hormone specific ß subunit. Purified FSH administered alone or in combination with luteinizing hormone (LH), has been used to induce a superovulatory response. The results with these hormones or with pregnant mare serum gonadotropin (PMSG), which contains intrinsic FSH and LH activity, have been variable. The use of recombinant bovine FSH (rec.bFSH), which is guaranteed to be free of LH, and which is homologous to the species in which it is applied most frequently, may improve superovulation results. Furthermore, bovine FSH is difficult to purify in substantial quantities from bovine pituitaries (Wu *et al.*, 1993). Rec.bFSH therefore may provide sufficient material to allow for structure-function studies by epitope mapping (Geysen *et al.*, 1984; Westhoff *et al.*, 1994).

cDNA's of bovine a subunit (Erwin *et al.*, 1983; Nilson *et al.*, 1983), as well as cDNA's of bovine FSH ß subunit (Esch *et al.*, 1986; Maurer & Beck, 1986) have been isolated.

Recombinant FSH has been produced in chinese hamster ovary (CHO) cells for the human (Keene *et al.*, 1989; Van Wezenbeek *et al.*, 1990; Roth *et al.*, 1993) and the ovine (Mountford *et al.*, 1994) species, whereas for the bovine species recombinant FSH has been produced in CHO cells and in

transgenic mice (Greenberg *et al.*, 1991). Rec.bFSH has also been produced in mouse epithelioid cells (Chappel *et al.*, 1988) and has been applied for superovulation in cattle (Looney *et al.*, 1988; Wilson *et al.*, 1989, 1993).

The baculo virus expression system is based on the infection of insect cells with a recombinant baculovirus (L.A. King and R.D. Possee, 1992) and is increasingly used for production of heterologous proteins. Insect cells have the glycosylation apparatus capable of synthesis of high mannose or hybrid type carbohydrates, as well as simple O-linked chains, and recombinant proteins can be expressed with much higher efficiency as compared with the chinese hamster ovary or COS cell system (Chen *et al.*, 1991). The baculovirus expression system has been used to produce amongst others the a subunit of hCG (Nakhai *et al.*, 1991a,b), the a subunit of carp gonadotropin (Huang *et al.*, 1991; Chen and Bahl, 1991), the ß subunit of hCG (Chen *et al.*, 1991; Sridhar and Hasnain, 1993; Sridhar *et al.*, 1993; Nakhai *et al.*, 1992; Jha *et al.*, 1992), hCG (Chen and Bahl, 1991; Nakhai *et al.*, 1992), the receptor for human FSH (Christophe *et al.*, 1993) and, quite recently, human FSH (Lindau-Shepard *et al.*, 1994; Dias *et al.*, 1994) (Table 1). Co-expression of two, or more, proteins by the baculovirus expression system has been achieved for instance by construction of a multiple expression transfer vector containing two, or more, foreign genes each of which is under the control of a copy of the p10 or polyhedrin promoter. Such expression vectors have been applied to the production of 2 totally unrelated proteins, for instance luciferase and hCG ß (Hasnain *et al.*, 1994), but also to the production of 3 or 4 closely related proteins, which may be assembled *in vivo* to complex structures (Belayev and Roy, 1993). Such a system might also be used for co-expression of FSH a and FSH ß, including the bovine forms. However, the synthesis of protein complexes has also been accomplished by co-infection of insect cells with two different recombinant viruses. This has been applied to bluetongue virus proteins (French, Marshall & Roy, 1990), hCG (Chen & Bahl, 1991) and hFSH (Lindau-Shepard *et*

al., 1994). Here we report for the first time the synthesis of bovine FSH in insect cells, by co-infection of cells with two recombinant viruses carrying the genes of bFSHα and bFSHß, respectively. This bFSH appears to be active in at least three different bioassay systems. Production in insect cells of only bFSHα was about 10 times higher than of only bFSHß, but co-infection of the two recombinant viruses resulted in production of heterodimer at a level comparable to that of bFSHα alone. A similar effect has been observed with the production of recombinant ovine FSH in Chinese hamster ovary cells (Mountford *et al.*, 1994), and of recombinant hCG in monkey cells (Reddy *et al.*, 1985).

Up to now no reports have been presented describing baculo expression of bovine FSH.

A surprising effect, obtainable by expressing bovine FSH in baculovirus based systems, is that very high biological activity is found, as demonstrated both in a heterologous system containing human FSH receptors, and in a homologous system containing bovine immature oocytes. It appears that the biological activity of baculo-derived rbFSH is at least as high as native FSH purified from pituitaries, or as rbFSH produced in higher eukaryotic cell systems.

This leads directly to an application in humans, especially in those cases in which administration of FSH needs to be carried out only a limited number of times, or in which the application can be carried out *in vitro*. Furthermore parts of the rbFSH molecule may act as an FSH antagonist and therefore can be used as a male contraceptive. This will only be possible if (fragments of) bovine FSH produced in baculovirus systems will not be immunogenic, and can therefore be used in humans without restrictions. Alternatively, bFSH or fragments of it may be used for vaccination against FSH as a means of contraception in the male. In the human this could be an attractive alternative for the use of hFSH, because a heterologous hormone (or part of it) may be better immunogenic than the homologous hormone.

For the bovine species the results of the oocyte maturation inhibition test lead to application in superovulation treatments in the bovine, where it can act as a substitute for Pregnant Mare Serum Gonadotropin (PMSG) or other hormones with FSH activity, in the treatment of reproductive problems such as anoestrus incomplete follicle development etc. It can also be used in *in vitro* experiments, for instance for the purpose of *in vitro* maturation and fertilization of oocytes. The biological activity of baculo-derived rbFSH in a rat-Sertoli-cell assay and a Y_1 cell assay indicates that this biological activity most likely is not species specific. Applications therefore can be expected in other species than the human, bovine or rat, both *in vivo* and *in vitro*.

The invention further provides to tailor the degree of sialylation, and thus the metabolic clearance rate and *in vivo* biological activity of FSH, by cloning the transsialydase-gene into the subunit-gene(s) containing baculo-vector. This may allow for addition of neuraminic acid to the glycan cores of rbFSH, and thus for increased biopotency.

Another part of the invention provides for fusion of (parts of) the bFSHß- and bLSHß-gene in order to tailor chimaeric hormones with a fixed ratio of FSH to LH bioactivity.

It will be understood that these kind of applications and embodiments lie within the scope of the present invention. Thus, where FSH is used in the present application this must be read as including fragments and/or derivatives thereof. It will also be clear that the exemplified vectors and/or regulatory elements are only examples and that other vectors capable of expression in insect cells will be suitable as well, as will other regulatory elements. The cloning techniques are also known in themselves and may be varied. The exemplified cell line is a well known and often used insect cell line. Other cell lines capable of being transfected by the vectors of the invention will also be applicable. Culture media for the transfected cells can be suitably selected by

the person skilled in the art. Once bovine FSH has been expressed it is known how to isolate it from the culture. Once isolated and/or purified pharmaceutical preparations can easily be formulated using the knowledge obtained with other recombinant or isolated gonadotropins.

The invention will be explained in more detail in the following experimental part.

Experiments

Materials and methods

Viruses and cells

Autographa californica Nuclear Polyhedrosis Virus (AcNPV) and recombinant virus stocks were propagated in Spodoptera frugiperda clone-21 (Sf21) cells grown as monolayers in TC100 medium (GIBCO-BRL), supplemented with 10% fetal calf serum plus antibiotics. For cotransfection, Sf21 cells were grown in Grace medium (Grace, 1962), supplemented with 10% foetal calf serum plus antibiotics. For immunological assays like RIP or IPMA and for protein production, Sf21 cells were grown in Sf900 serum-free medium (GIBCO-BRL) plus antibiotics. In order to reduce the background of wild type virus, modified AcNPV in which the p10 gene was exchanged for a synthetic and unique *BSU*36I restriction site was used for cotransfection (Martens *et al.*,1994). After homologous recombination between wild type virus and the transfer vector, circular recombinant viral DNA will be formed, which can infect Sf21 cells. Non-circular DNA is not infectious, and therefore background will be reduced. However, due to non-homologous recombination, background percentage will be reduced from 95% to 70% only (Martens, 1994).

Enzymes and chemicals

Restriction enzymes and phage T4 DNA ligase were purchased from Biolabs (USA) and used as recommended by the supplier. ^{35}S methionine was obtained from Amersham UK. VenR ^{TM}DNA polymerase was from Biolabs (USA).
All cloning procedures were carried out essentially according to Sambrook *et al.* (1989).

Plasmids, and construction of transfer vectors

The cDNA coding for bFSHa was purified after double digestion of the plasmid bov Alpha-pSP64 #1 (Leung *et al.*, 1987) with *Nco* I plus *XBa* I. The DNA of 554 bp's contained a signal sequence of 72 bp at the 5' end , and an untranslated

region of 188 bp at the 3' end. It was cloned into the unique *Nco* I and *XBa* I sites of vector pARKh1 which is a derivative of transfer vector pAcAs3 (Vlak *et al.*, 1990). The *Nco* I site contained an ATG codon which coïncided exactly with the start of the signal sequence of bFSHα. Correct insertion with respect to the p10 gene of bFSHα in the vector was confirmed by extensive restriction enzyme analysis and sequencing (dideoxy method), and the selected transfer vector was designated pDWa9.1 (Fig.1).

DNA coding for bFSHß was obtained by amplification of the relevant region of Bov FSHbeta pGEM3 (Maurer and Beck, 1986) by the polymerase chain reaction (PCR). A 39-cycle amplification was performed with Ven DNA polymerase. The sequences of the synthetic oligonucleotides used in PCR reactions were as follows (5' 3'): 1, C C T G A G A G A T C T A T C A T G A A G T C T G T C C A G T T C T G; 2, G A G G G A T C C A G A T C T A G A G G A T T T A G G T G A C A C T A T A.

Primer 1 introduced a *BspH* I restriction site by changing the sequence A G G A T G A A G into A T C A T G A A G, which allowed cloning of the bFSHß-cDNA on the ATG at the start of the signal sequence. Primer 2 introduced a combined *Bgl* II/*XBa* I restriction site and a SP6 flag at the 3' end of bFSHß-cDNA.

After PCR, the bFSHß-cDNA of 1.5 kb length was purified by electrophoresis in a 4% agarose gel, and doubly digested with *BspH* I/*Stu* I. A 348 bp DNA fragment was isolated and cloned into the unique *Nco* I and *Stu* I sites of the vector $pARKh_1$. The recombinant plasmid was termed $pDW\ss_1$.

Vector $pARKh_1$ was derived from vector $pAcAs_3$ (Vlak *et al.*, 1990). $pAcAs_3$ is a transfer vector of 9809 bp, containing the baculovirus p10 promoter, directly flanked by a unique *BamH* I site. The nucleotide sequence around this *BamH* I site was first modified by PRC in such a way, that an ATG start codon was formed; the resulting plasmid was called pAcMo8 (Vlak & van Oers, 1994). Further modifications by PCR introduced a multiple cloning site (MCS) containing a *Nco* I site, followed

by *Bgl* II, *Xba* I, *Pst* I and *BamH* I. This plasmid was called pPA_I. A synthetic MCS plus hybrid envelope glycoprotein of hog cholera virus (E_1) plus 3 stop codons were inserted by cloning *Bgl* II + blunted *PSt* I of pPEh8 (van Rijn *et al.*, 1992) into *Bgl* II + blunted *BamH* I of pPA_1, resulting in transfer vector $pARKh_1$. Hybrid E_1 contains a unique *Stu* I site, which allowed for the exchange of E_1 for bFSHß. bFSHß was cloned into $pARKh_1$ in two parts. The 5' part was obtained by PCR, and the 3' part by regular DNA isolation from miniprep plasmid DNA (348 bp DNA fragment; see above) of Bov FSHß $pGEM_3$. This strategy was chosen in order to minimize possible errors, which can be introduced by amplification via PCR.

Plasmid BovFSHß $pGEM_3$ was digested with *Stu* I and *Bgl* II. Because of methylation of the *Stu* I restriction site, this site was only partially digested. A 1106 bp fragment was isolated by excision from a 4% agarose gel and purified according to standard techniques. This fragment was ligated into the *Stu* I/*BamH* I sites of vector pDWß1. Before transformation, the ligation mixture was digested with *Bgl* II for the purpose of background reduction. The resulting recombinant plasmid pDWß3.1 now contains a 1454 bp bFSHß fragment consisting of a 57 bp 5' fragment encoding the signal sequence, a 330 bp fragment coding for bFSHß, and a 1067 bp 3' untranslated region, and it had an ATG codon exactly at the start of the signal sequence (Fig.1).

The correct orientation of the bFSHß gene with respect to the p10 promoter was confirmed by extensive restriction enzyme analysis and by sequencing the ligation regions.

Construction of baculovirus recombinants expressing bFSHα or bFSHß

Viral AcNPV DNA isolated from extracellular budded virus particles (0.15 µg) was completely digested with *BSU*36I (30 U/µg/h, for 5 hours). DNA was purified by standard procedures and dissolved in 15 µl 1mM Tris/0.1mM EDTA buffer (pH 8.0; TE buffer).

Confluent monolayers of Sf21 cells (7.5 to 8 x 10^6) grown in 9 cm diameter petri dishes were cotransfected with 0.1 µg of digested viral AcNPV DNA, and 2 to 3 µg of transfer vector DNA by the calcium phosphate precipitation technique described by Summers and Smith (1987).

After transfection, cells were washed with TC-100 medium, and covered with 16 ml of a TC100 agar overlay, containing 60 µg Bluo-Gal (GIBCO-BRL) per ml. Cells were grown for 4 to 6 days, and blue plaques were picked and were further plaque purified in M6 plates (Costar). Plaque purification was repeated until no more white plaques of wild type virus could be observed. Purified blue plaques were used to infect confluent monolayers of Sf21 cells in M24 plates (Costar). After 4 days, the cells were fixed and tested for expression of bFSH subunit by an immune peroxidase monolayer assay (Wensvoort *et al.*, 1986), after incubation with a 1:1000 dilution of polyclonal rabbit antiserum against either bFSH (a gift from J. Closset and G. Hennen) or oFSH (H. Westhoff), or bFSHß (USDA-5-pool, a gift from D. Bolt). Media were tested for presence of bFSH subunit by ELISA in M96 microtiter plates (Costar); 10 µl of medium was coated (0.05 M carbonate buffer, pH 9.65/1 hr/37°C) onto the bottom of a well and incubated with rabbit polyclonal antisera against either bFSHα or bFSHß (A.F. Parlow). Plaque-purified viruses both for bFSHα and bFSHß were selected, and were used for preparation of virusstocks. After double infection with a recombinant virus containing bFSHα plus a recombinant virus containing bFSHß, media were analyzed for bFSH heterodimer in an antigen capture assay (ACA) based on trapping of bFSHαß in a 96 wells plate, coated with a commercial monoclonal antibody (MCA, code ME.112) against human FSHß (MBS, Maine, USA) This MCA was shown to crossreact with bFSHß. The wells were then incubated with rabbit anti-bFSHα polyclonal antisera (A.F. Parlow) followed by HRPO-conjugated rabbit-anti-guinea-pig-IgG (RAGPPO, Dako, Denmark) and substrate solution (with tetra mehyl benzidine as the chromogen). Reference preparations bFSHα, bLHα, bFSHß, bFSHαß were a gift from D. Bolt and A.F.

Parlow, and bFSHαß, bFSHα and bLHα were a gift from J. Closset and G. Hennen (Univ. of Liège, Belgium).

DNA analysis

Viral and cellular DNAs were isolated from Sf21 cells infected with wild type and recombinant AcNPV viruses as described by Summers and Smith (1987). Restriction enzyme-digested viral and cellular DNAs were analyzed by electrophoresis on a 4% agarose gel, and it was shown that the DNA sequences encoding bFSHα and bFSHß were correctly inserted in the p10 locus of baculovirus.

The nucleotide sequence of the junctions between bFSH subunit and transfer-vector DNA were determined by the dideoxy chain termination method with T_7 DNA polymerase (Pharmacia) and primers (5' 3') pAcAs-upi (CAACCCAACACAATATATT) and pAcAs-rupi (GGTTACAAATAAAGCAATAGC).

Radiolabeling and analysis of proteins

Radiolabeling and analysis of recombinant proteins with ^{35}S methionine (Amersham, UK) were done as described by Hulst *et al.* (1993). For immunoprecipitation of bFSHß, either monoclonal antibody against human FSHß (ME.112, commercially obtained from MBS, Maine, USA) or polyclonal guinea pig anti-bFSHß antiserum (A.F. Parlow) were used, whereas for bFSHα polyclonal guinea pig anti-bFSHα (A.F. Parlow) was used. (Monoclonal ME.111 against hFSHα was also used, but did not cross-react with bFSHα.)

ELISA and antigen capture assay (ACA)

bFSHα and bFSHß subunits, expressed by recombinant viruses, were detected by specific ELISA systems. M96 plates (Costar) were coated with medium (maximally 10 µl /well) collected from Sf21 cells which were infected with either AcNPVα3.4 or AcNPVß1.4. Coated wells were then incubated (1h/37°C) with 1:1000 diluted polyclonal guinea pig anti-bFSHα or -bFSHß antisera (A.F. Parlow). Bound immunoglobulins were detected with 1:500 diluted rabbit-anti-guinea-pig-IgG coupled to horseradisch peroxidase (RAGPPO, Dako, Denmark), and

tetramehylbenzidine as substrate. Optical density was measured at 450 nm. Purified pituitary bFSHα (Closset and Hennen) and bFSHß (USDA-bFSH-beta; Bolt) were used as reference preparations (1, 10, 20, 40, 80 ng/well) for quantitative measurement. Bovine FSHαß heterodimer expressed after double infection (at MOI>10) with recombinant viruses AcNPVα3.4 plus AcNPVß1.4 was detected by antigen capture assay (ACA) as described by Wensvoort *et al.* (1988).

Briefly, monoclonal antibody against human FSHß (a commercial preparation of MBS, Maine, USA, crossreacting with bFSHß and bFSHαß) was used as capture antibody at a dilution of 1:100 (1µg/100µl/well) by coating it on a M96 well (1h/37°C). Medium (maximally 100 µl/well) harvested from doubly infected Sf21 cells was incubated in coated wells (1h/37°C) and bound bFSHαß was detected by sequentially incubating with 1:1000 diluted polyclonal guinea pig anti bFSHα (A.F. Parlow)(1h/37°C) and RAGPPO (1h/37°C).

The substrate reaction was as described for the ELISA. Purified pituitary bFSHαß (USDA-bFSH-I-2, D. Bolt, or bFSH from J. Closset and G. Hennen) was used as reference preparation (1-80 ng/well) for quantitative measurements. (It should be noted that measurement of bFSHαß in this system may lead to underestimation because of blocking of capture antibody by free bFSHß subunits.)

Time course of production of subunits or heterodimer

The time courses of production of rec.bFSHα, rec.bFSHß and rec.bFSHαß were determined essentially as described by Hulst *et al. (1993)*. Media were clarified by centrifugation for 10 minutes at 1000 x g, and were analysed by ELISA (subunits) or ACA (heterodimer).

Y_1-cell bioassay

Y_1 mouse adrenal cells, stably transfected with cDNA for the human FSH receptor (coupled to the gene for resistance to methotrexate) were kindly donated by ARES, Serono, Rome, Italy. Those cells repond to FSH stimulation with cAMP

accumulation, progesterone synthesis and a change in cell morphology. Unstimulated cells grow flat on the surface, but after addition of a cAMP stimulating agent the cells round off. This change in cell-morphology is maximal after two to three hours and disappears after approximately 7 hours. The optical density (O.D.) of the cells changes after rounding off and can be measured with an ELISA reader, at 405 nm. The rounding off shows good correlation with cAMP accumulation (Westhoff *et al.*, 1994). Cells were plated in M96 plates in Ham's F10 medium (GIBCO) supplemented with 2 mM l-glutamine. The incubation with FSH was carried out in Ham's F10 medium, and O.D. was measured after 0.5, 1, 2, 3, 4, and 6 h incubation. At 2 and 4 hours the rounding off was also determined light-microscopically by the naked eye. One hundred µl aliquots of media were harvested at 2 hrs, for cAMP determination (cAMP ^{3}H assay systems, Amersham TRK 432, UK). The minimal dose of bovine FSH (USDA-bFSH-I-2) giving a significant response in the Y_1 cell assay is 4 ng/ml, ovine FSH (oFSH, NIADDK-oFSH-16, AFP-5592C) 30 ng/ml, and of porcine FSH (pFSH, NIH-FSH-P-1) 200 ng/ml.

Rat Sertoli-cell bioassay

The rat Sertoli-cell bioassay was done as described by Oonk *et al.* (1985) and Oonk & Grootegoed (1987). Culture media were harvested, and analyzed for cAMP concentrations (cAMP ^{3}H assay systems, Amersham TRK 432, UK)

Oocyte-maturation inhibition bioassay

In vitro maturation of isolated oocyte-cumulus complexes can be inhibited by a amanitin containing culture media in combination with small doses of FSH. Bovine oocyte-cumulus complexes were isolated form fresh slaughterhouse material, and tested for maturation inhibition (i.e., absence of germinal vesicle break down, GVBD) by FSH according to Hunter and Moor (1991).

Affinity chromatography and analysis of immunoactivity of rbFSH

Recombinant bFSH was purified by affinity chromatography, using a monoclonal antibody -against human FSHß subunit- coupled to CNBr activated Sepharose (Sepharose 4B, Pharmacia). 1.5 Gram of Sepharose 4B was washed and allowed to swell as recommended by the manufacturer. Monoclonal antibody (Mab) against human FSHß (code ME.112, Maine Biotechnology Services, Inc., Portland, ME, USA), 9 ml containing 9 mg of purified lgG1, was dialysed overnight against 1 L of couplingbuffer (0.1M $NaHCO_3$/0.5M NaCl pH 8.3). The resulting Mab solution (8 ml) was incubated with 5 ml of swollen gel (overnight, 4°C, end-over-end mixing). Coupling efficiency by A280 measurement was 98%.

After washing with coupling buffer, 0.1 M Tris pH 8.0, 0.1 M acetate/0.5 M NaCl pH 4 and 0.1 M Tris/0.5 M NaCl pH 8 respectively, the coupled Mab was incubated with 130 ml sterile (0.2 µ filter) Sf900 insect cell culture medium (Gibco) containing rec. bovine FSH αß heterodimer (approximately 1 µg/ml by immunoassay).

As a control experiment, 2 ml of coupled Mab was mixed with 30 ml sterile (0.2 µ filter) Sf900 insect cell culture medium containing rec. bovine FSHα had been harvested at 72 hours after infection. Binding reactions were allowed to proceed for 24 hours at 4°C, under gentle shaking.

The sediment was separated by centrifugation (10'/500 g/4°C) and supernatants were kept apart for determination of binding efficiency. Columns were packed in pasteur pipets with bed volumes of approx. 2 ml and 1.5 ml for rb FSHαß was eluted stepwise with sterile cold (ice) PBS (10 ml), and 0.1 M glycine HCl/0.1 M NaCl buffer with pH 4.0 (6 ml), pH 3.5 (6 ml), pH 3.0 (7 ml), pH 2.5 (6 ml) and pH 2.0 (5 ml) respectively. 1 ml fractions were collected on ice, and pH was immediately neutralised with 3 M Tris.

All fractions were stored at -20°C until assayed.

Analysis of immunoactivity was performed by antigen capturing assay (ACA) whereas bioactivity was determined by

two *in vitro* bioassays, i.e. Y_1 cell assay and Sertoli cell assay. Furthermore, fractions were concentrated (10X) on 'Centricon 10 or Centricon 30 filters (Amicon, Inc. Beverly, MA, USA) and analysed for purity and protein content by SDS-Page (12%) under non-reducing conditions and staining with silver.

Results

Construction, selection and characterization of recombinant viruses expressing bFSHα or bFSHß

Transfer vectors pDWα9.1 and pDWß3.1 were constructed as depicted in Fig. 1.

S f21 cells were cotransfected with pDWα9.1 or pDWß3.1 and wild-type (wt) AcNPV/MO_{21} DNA isolated from extracellular virus particles. In this wt virus, the p10 coding sequence is replaced by a *BamH* I oligonucleotide linker with a unique *BSU*36I recognition site (Martens *et al.*, 1994). This allows for an increased proportion of recombinants after eliminating the parental virus by linearization.

Polyhedrin-positive plaques expressing ß-galactosidase were isolated and analyzed for expression of bFSHα or bFSHß by immunostaining of cells with polyclonal rabbit antisera, and by ELISA of culture media with polyclonal guinea pig antisera (A.F. Parlow). One plaque-purified bFSHα virus (AcNPV/α3.4) and one plaque-purified bFSHß virus (AcNPV /ß1.4) were used to prepare virusstocks with a tissue culture dose of infection (TCID) of approximately 7 and 8, respectively.

The α and ß expression products were further characterized by radio immuno precipitation (Fig. 2a+b). bFSHα, which was precipitated from the medium of Sf21 cells infected with AcNPVα3.4, migrated as a single band with a molecular mass of approx. 18 kD (Fig. 2a, lane 4). Cell lysates showed a variety of labeled bands, which may be due to the use of polyclonal instead of monoclonal antibodies (lane 3). Monoclonal antibody against hFSHα (MBS, Maine, USA) did not precipitate any bFSHα, which was expected as this antibody

did not show cross reaction with bovine a subunit in the ELISA.

bFSHß, which was precipitated from the medium of Sf21 cells infected with AcNPV/ß1.4, migrated as a doublet, with a molecular mass of 15-16 kD, both with polyclonal antisera (Fig 2b, lane 4)(guinea pig anti-bFSHß, A.F. Parlow) and monoclonal antibody (anti hFSHß, MCS, Maine, USA)(lane 9).
In cell lysates a doublet of slightly higher molecular weight was observed with both antibodies (lanes 3 and 8).

Expression and secretion of bFSHα and bFSHß

The levels of expression of bFSHα, bFSHß and bFSHαß in the medium of infected Sf21 cells were determined at different time intervals after infection, and the levels in Sf21 cell lysates were determined at 162 hours after infection, by specific ELISA systems and ACA (Fig. 3). The majority of bFSHα, bFSHß and bFSHαß was secreted into the medium, and only very small amounts were found in the cell lysates. Levels of bFSHα in medium were approximately 10 times higher than levels of bFSHß, whereas levels of bFSHαß were intermediate. Reference preparations used were bLHα:AFP.3IIIA (Parlow), bFSHß: USDA-bFSH-beta-subunit (Bolt) and bFSHαß: UCB-i028 (Hennen/Closset). The maximum concentration of bFSHα was 1.1 $\mu g/10^6$cells/0.5 ml at 48 hours after infection (p.i.). For bFSHß the maximum was 0.13 $\mu g/10^6$cells/ 0.5 ml at 72 hours p.i., and for bFSHαß the maximum was 0.65 $\mu g/10^6$cells/0.5 ml at 92 hour p.i. In cell-lysates, bFSHα- and bFSHß-concentrations were below the detection limit of the assay, and bFSHαß-concentration was less than 0.01 $\mu g/10^6$cells.

Y_1-cell bioassay

In vitro bioassays were done on 5 ml aliquots of media (TC100) containing bFSHα and bFSHß; these media were first concentrated (20 x) by speedvac, and then mixed and incubated (16h/27°C) according to Nakhai *et al.* (1992).

Concentrated media containing bFSHα, bFSHß or bFSH(α+ß) were serially diluted and added to Y_1 cells. It appeared that

no change in morphology could be observed with either bFSHα or bFSHß, but distinct responses could be observed with bFSHαß up to a 1:20 dilution of concentrated media.

In another experiment, Y_1-cell *in vitro* bioassays were done on SF900 media (serumfree) of Sf21 cells infected with either AcNPVα3.4 or AcNPVß1.4 alone, or with AcNPVα3.4 plus AcNPVß1.4. These media were directly diluted, without prior concentration by speedvac.

It appeared that media containing only bFSHα or bFSHß did not induce a change in cell morphology, but media from cells infected with AcNPVα3.4 plus AcNPVß1.4 showed very clearly FSH-specific responses up to a dilution of 1:800, which corresponds to a biological activity of 8-15 $IU.ml^{-1}$ (ref.prep. USDA-bFSH-I-2; 854 $IU.mg^{-1}$). This indicates that the yield of bFSHαß after double infection was approximately 800 times higher than after reassociation of separately produced bFSH subunits; however, there may have been also a non-specific inhibitory effect of concentrated TC100 medium on Y_1 cells.

Media harvested from Y_1-cell cultures were analyzed for cAMP. It appeared that Y_1-cells which were incubated with baculomedia from doubly infected Sf21 cells showed dose-dependent cAMP responses.

Comparison with a (freshly prepared) reference preparation of bFSH (USDA-bFSH-I-2), gave a bioactivity of 20-24 IU/ml, whereas bioactivity of both single subunit-containing media was zero.

Rat-Sertoli-cell assay

Bioactivity of rbFSH media as determined in a rat-Sertoli-cell *in vitro* bioassay by comparison with USDA-bFSH-I-2 as a reference preparation, varied between 4 and 9 $IU.ml^{-1}$; again single subunit-containing media were negative. Maximal stimulation however of rbFSH was lower by a factor 2 to 4 as compared to USDA-bFSH-I-2. This may be due to differences in glycosylation between pituitary and recombinant bFSH.

Oocyte-maturation inhibition assay

rbFSH culture media was tested at a dilution of 1:25 in a bovine oocyte-cumulus *in vitro* bioassay, with bovine FSH from Sigma (25 S_1 U/vial) as a reference preparation. A bioactivity for rbFSH was found of 6.3 $IU.ml^{-1}$, whereas for rbFSHα- and rbFSHß-subunits no bioactivity was observed (Fig.4).

Affinity chromatography and analysis of immunoactivity of rbFSH

As can be seen from figures 5 and 6, the immunoactivity of the purified rbFSH corresponded fully with the biological activity as measured in the Y_1 cell assay and the Sertoli cell assay.

Bioactivity before affinity chromatography was 6.4 or 4.2 lU/ml (Y_1 cell assay and Sertoli cell assay, respectively) whereas immunoactivity was 2.5 µg/ml (ACA). Total amount of rbFSH therefore was 833 or 546 lU (bioassay) and 325 µg (immunoassay), respectively. The combined amount of rbFSH of all fractions after affinity chromatography was 25 lU or 50 lU (Y_1 cell assay and Sertoli cell assay, respectively), or 23 µg (ACA). Percentage recovery after affinity chromatography therefore was 3.0% (Y_1), 9.1% (Sertoli-cell) and 7.1% (ACA), respectively.

Discussion

Production levels of rec.bFSHα and rec.bFSHß in our system are comparable with gonadotropin subunit levels obtained in the baculosystem which were published previously (Table 1). These levels however are very much dependent on the type of assay and the reference preparation which were used. Sofar we have not done purification of rbFSH subunits or hormone, and specific (bio)activityper unit of weight is based on ELISA in which purified hormone-subunits were used as reference preparations. It has been mentioned in the literature that specific activity of rhFSH can vary between 10.000 and 40.000 IU mg^{-1}, depending on the method of protein recognition and/or the use of various protein standards (Mannaerts *et al.*, 1991).

In our study, specific activity of rbFSH expressed in terms of bFSH (USDA-bFSH-I-2, 854 $IU.mg^{-1}$) bioactivity (Y_1 cell assay/cAMP) and bFSH (UCB io58) immunoactivity (ACA) is approximately 20.000 $IU.mg^{-1}$.

More accurate determination of S.A. however awaits further purification of rbFSH and direct estimation of protein content. From thses data it will be possible also to calculate the ratio of bioactivity to immunoactivity of rbFSH.

Bioactivity of glycoprotein hormones is dependent also on type and extent of glycosylation as has been demonstrated for rhCGß (Sridhar and Hasnain, 1993). In order to relate bioactivity of rbFSH to degree and type of glycosation, it will be necessary to analyse glycosidic side-chains or this hormone. This also may reveal possible microheterogeneity, as has been demonstrated for rhFSH (De Boer and Mannaerts, 1990). The observed variation in bioactivity between different bio-assays (cAMP production of Y_1 cells, morphological changes of Y_1 cells, cAMP production in rat-Sertoli-cells, maturation inhibition of bovine oocytes) may be explained by differences in glycosylation between pituitary and recombinant bFSH.

Untill now, bovine recombinant FSH has been produced only in mouse epitheloid cells (Chappel *et al.*, 1988) and in transgenic mice (Greenberg *et al.*, 1991), although reference

was made also to CHO cells (Greenberg *et al.*, 1991, commercial preparation from Genzyme Corp.). Reports about application of rbFSH for superovulation in cattle do not give any specification of the rbFSH used (Looney *et al.*, 1988; Wilson *et al.*, 1988; Wilson *et al.*, 1993), although it apparently is from commercial origin.

Most likely all these rbFSH products were based on the same subunit cDNA's as were used in our baculo-expression system. Sofar, the only rFSH which has been produced in the baculovirus system, is human FSH (Lindau-Shepard *et al.*, 1994; Dias *et al.*, 1994). The cDNA that was used for hFSHa subunit consisted of a 51 bp untranslated 5' region, a 72 bp signal sequence, a 276 bp sequence of the a subunit, and a 222 bp untranslated 3' region. In contrast, the cDNA of the ß subunit contained the minimal contiguous hFSHß sequence, including the leader sequence but without untranslated regions at either the 5' or 3' end. It is our feeling that the untranslated 3' region which we have used in the cDNA of the bovine FSHß subunit, may have contributed to its stability and to a high production level.

To further illustrate this phenomenon the posttranscriptional regulation of bFSHβ subunit mRNA is discussed below

FSHβ mRNA

The FSHβ subunit is encoded by a single gene in species studied, which has been characterized in the human, rat and cow, and contains three exons and two introns (reviewed by Haisenleder et al. 1994). FSHβ subunit biosynthesis most likely is a rate limiting step in FSH heterodimer assembly and secretion (Greenberg et al., 1991). The FSHβ mRNA nucleotide and polypeptide amino acid sequences are highly conserved between species (approx. 80%). In rats and cows, only one mRNA (of approx. 1.7 kb) has been demonstrated, but the human FSHβ gene produces four mRNA size variations. The different mRNA sizes appear to be due to the use of two different transcription start sites and two different polyadenylation

sites, but it is unknown if all four mRNA transcripts are translated or hormonally regulated. The biosynthesis and secretion of LH and FSH are under the control of multiple hormones: GnRH, which is released from the hypothalamus in a pulsatile manner, sex steroid hormones and the gonadal protein hormones inhibin, activin, and follistatin. The latter have preferential effects on FSH; inhibin and follistatin decrease FSHβ mRNA levels and FSH secretion, whereas activin is stimulatory. Follistatin binds activin with high affinity, blocking stimulation of FSH secretion, and inhibin with lower affinity.

Stability of FSHβ mRNA

Inhibin and follistatin appear to repress steady state FSHβ mRNA levels at least in part by reducing the stability of FSHβ transcripts (Dalkin *et al.*, 1993; Carrol *et al.*, 1991). In rats, the pulsatile administration of GnRH stimulates FSHβ gene transcription, while estrogen inhibits FSHβ mRNA transcription in vivo. In contrast, the ability of testosterone to elevate FSHβ mRNA levels in the presence of a GnRH antagonis is independent of any influence on gene transcription, and presumably represents a post-transcriptional effect on FSHβ mRNA stability (reviewed by Haisenleder *et al.*, 1994; Mercer & Chin, 1995). Similarly, the gonadal peptide activin enhances FSHβ mRNA expression in rat pituitary cell cultures, in part by increasing the half-life of the FSHβ transcript over 2-fold (Carrol *et al.*, 1991).

FSHβ mRNA 3'UTR

A common feature of FSHβ genes is an extremely long 3'UTR (1kb, 1.2 kb and 1.5 kb in the rat, bovine and human genes, respectively). This compares to LHβ- and TSHβ-mRNA which have a total length (including 3'UTR) of approximately 700 bp (Maurer and Beck, 1986).

There are five highly conserved segments within the long 3'UTRs of the rat, human and bovine FSHβ genes. Apart from this observation, sequences within the 3'UTR of several genes have

been shown to be important in determining RNA stability (reviewed by Gharib et al., 1990).

Removal of the majority of the 3'UTR from the ovine FSH-β subunit cDNA insert dramatically enhanced the accumulation of oFSHβ-mRNA transcripts in COS cells, indicating a role for this region in regulating mRNA stability. A similar effect is seen in stably transfected CHO cells, although a corresponding effect on oFSHβ mRNA translation is not found, possibly reflecting translational inefficiency of β subunit mRNA (Mountford *et al.*, 1994). The significance of this 3'UTR of FSHβ mRNA is presently unkown, but it has been speculated that it may play a role in determining FSHβ mRNA stability. This is supported by studies showing that elements in the 3'UTR can regulate mRNA in other cell systems (Haisenleder *et al.*, 1994).

AU-rich regions

Of particular interest is the presence of 6 copies of the pentanucleotide AUUUA within the reported 3'-UTR sequence of bovine FSHβ (in the ovine sequence also 6 of such motifs have been found; Mountford *et al.*, 1992). There is compelling evidence to suggest that this element plays a critical role in the destabilization of a number of short-lived cellular mRNAs encoding lymphokines and proto-oncogenes (Cleveland and Yen, 1989). These so-called AU rich sequences, when inserted into 3'UTR of a normally stable mRNA, have a destabilizing effect (Ross, 1988) and cause selective degradation of transiently expressed messengers (Shaw and Kamen, 1986).

These motifs have been found in highly labile mRNAs such a C-fos, or granulocytemonocyte colony-stimulating factor FM-CSF, and resemble the AU-rich motifs in the 3'UTR of the labile human LdhC (testis specific isozyme of lactate dehydrogenase) mRNA (Salehi-Ashtiani & Goldberg, 1995).

Size of FSHβ-mRNAU

Porcine FSHβ subunit cDNA has been used for production of pFSHβ in the baculovirus expression system (Sato *et al.*, 1994, JP930071875). The cDNA used in this system was isolated by Kato (1988) and contained 929 basepairs, although Northern analysis showed a length of about 1.8 kb. The porcine FSHß gene which was cloned into a baculovirus contained only 436 bp, which consisted of a 18 bp signal sequence, a 327 bp FSHß gene and a 91 bp 3'UTR (Sato et al., 1994, JP930071875). The total sizes of porcine FSHβ- and FSHβ-mRNA reportedly were in the 2 kb range (Maurer & Beck, 1986). Nucleotide analysis of bovine FSHβ mRNA showed a total length of 1728 basepairs, excluding a several hundred nucleotide tract of poly A at the 3'terminus. Therefore, the 1067 bp 3'UTR of bovine FSH cDNA which we have used (van de Wiel *et al.*, 1995), is approximately ten times as long as the 3'UTR of procine FSHβ cDNA used by the Japanese group, and is very close to the total length of 1341 bp found by Maurer and Beck (1986). Most importantly it contains four of the six ATTTA sequences found in the full length 3'UTR, whereas the truncated porcine FSHβ 3'UTR described by Sato et al., JP930071875, (1994) contains no ATTTA sequence.

Relationship between size of FSHβ-cDNA and production level

The size of bovine FSHβ mRNA which was isolated and used for expression in the baculovirus system by Sharma, Dighe and Canerall (1993) has not been reported. Production levels of both subunites in the soluble fraction reportedly were approximately 120 ng/ml; no mention was made of production of FSH heterodimer.

Production levels reported for rpFSH in Sf 21 cells by Sato *et al.*, JP930071875 (1994) were approximately 0.1 µg/ml, although in Tn5 cells a production was reported of 1 µg/ml. Specific activity of this rpFSH as calculated from their data was 1250 IU/mg. In our bovine system we obtained production levels of 1-5 µg/ml; specific activity in the same *in vitro*

bioassay as used by Sato *et al.*, JP930071875 (1994) (OMI) was 7700 IU/mg.

As reported in the literature, levels of expression of recombinant proteins in insect cells may be too high, thus compromising posttranslational processing and excretion of the wanted protein into the culture medium (Scridhar *et al.*, 1993; Sridhar & Hasnain, 1993). High production levels of porcine LH receptor for instance resulted in intracellular accumulation and degradation of the product, with relatively low levels excreted into the medium (Bozon et al., 1995; Pajot-Augy *et al.*, 1995). We have now found that increasing the length of the 3'UTR of bFSHβ cDNA which we have used and thus increasing the number of ATTTA sequences, significantly increased the levels of excreted product, as compared to the results of Sato *et al.*, JP930071875 (1994).

Apparently, by selecting the length of the 3'UTR of FSH subunit cDNA, and thus choosing the number of specific ATTTA sequencs, one may selectively modify the stability of the corresponding mRNA, and modify the levels of the product that is excreted by the insect cells used.

Fig. 1

Scheme of the construction of transfer vectors pDWα9.1 and pDWß3.1. Arrows show the directions of transcription of the hsp70 (Lac Z), T7, Sp6 and p10 promoters. Ac, AcNPV DNA; p10, p10 promoter, hsp70, Drosophila melanogaster hsp promoter; SV_{40t}, SV_{40} transcription termination sequence, Lac Z, E. coli lac Z gene; B, *BamH* I; E, *EcoR* I; H, *Hind* III; X, *Xho* I, PCR, polymerase chain reaction; P, *Pst* I; N, *Nco* I; stop, stopcodons, S, *Sal* I; Bg, *Bgl* II; Sm, *Sma* I; Sa, *Sac* I; E_1, Hog cholera virus glycoprotein E_1, Amp, ampicillin resistance gene.

Fig. 2

Radio immune precipitation assay with polyclonal bFSHa antiserum (Parlow #5551791), polyclonal bFSHß antiserum (Parlow #899691), monoclonal antibody against hFSHß (code ME.112, MBS, Maine, USA) and monoclonal antibody against hFSHα (code ME.111, MBS, Maine, USA).

Culture media and cell lysates of Sf21 cells were analyzed after infection with AcNPV/$\alpha_{3.4}$, AcNPV/$ß_{1.4}$ or AcNPV/MO_{21} (control). Cells were labeled at 42 h after infection with 40 µCi of [^{35}S]methionine per ml for 6 h. Immunoprecipitates were analyzed by SDS-12% PAGE and visualized by autoradiography. A. bFSHα. B. bFSHß.

Lanes: 1 and 6, mol. weight markers (rainbow trout), M.W. x 10^3; 2 and 7, AcNPV/MO_{21} (wt) cell lysate; 3 and 8, recombinant AcNPV/($\alpha_{3.4}$ or $ß_{1.4}$) cell lysate; 4 and 9, recombinant AcNPV/($\alpha_{3.4}$ or $ß_{1.4}$) medium; 5 and 10, AcNPV/MO_{21} (wt) medium.

Polyclonal antisera were used in lanes 2-5, and monoclonal antibodies were used in lanes 7-10.

Fig. 3

Time course of production in Sf21 cells infected with AcNPV/$\alpha_{3.4}$(o-o) or AcNPV/$ß_{1.4}$(Δ -Δ) alone, or with AcNPV/$\alpha_{3.4}$ plus AcNPV/$ß_{1.4}$ (°-°).

ELISA concentrations of bFSHα and bFSHß, and ACA (antigen capture assay) concentrations of bFSHαß in culture media at 18, 24, 41, 48, 65, 72, 92, 96 and 150 h after infection are shown. Concentrations are expressed in µg (per 10^6 cells) of reference preparations bLHα-AFP-3111A, USDA-bFSH-beta and bFSH-io58.

Fig.4

Effect of rbFSH or subunits on GVBD in bovine cumulus-enclosed oocytes *in vitro*.

ON = oocyte nucleus stage (GV stage)
M = metaphase
D = diakynese
LD = late diakynese
T = telophase
C = negative control
+C = positive control (bFSH 0.25 $IU.ml^{-1}$)
α = rbFSHα
ß = rbFSHß
α+ß= rbFSHαß

Numbers on top of the bars indicate numbers of oocytes tested.

FIG. 5

Analysis of immunoactivity and bioactivity in a Y_1 cell assay of affinity purified rbFSH.

FIG. 6

Analysis of immunoactivity and bioactivity in a Sertoli cell assay of affinity purified rbFSH.

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CLAIMS

1. A method for the production of bovine follicle stimulating hormone, wherein a gene encoding an alpha subunit and a gene encoding a beta subunit of bovine follicle stimulating hormone are brought into an insect cell by means of at least one vector based on a baculo virus, wherein said resulting cells are cultured in a suitable medium and whereby bovine follicle stimulating hormone is recovered from said culture.

2. Recombinant bovine follicle stimulating hormone obtainable by a method according to claim 1 having a biological activity of at least 8000 I.U./mg in a Y_1 cell assay.

3. A recombinant Baculovirus vector or a corresponding recombinant Baculovirus comprising at least the gene coding for the alpha subunit of bovine follicle stimulating hormone.

4. A recombinant Baculovirus vector or a corresponding recombinant Baculovirus comprising at least the gene coding for the beta subunit of bovine follicle stimulating hormone.

5. A vector or a corresponding baculovirus according to claim 3 or 4 wherein at least one of the encoding genes comprises a stretch of untranslated nucleotides at the 3' end.

6. A vector or a corresponding baculovirus according to claim 5 wherein at least one of the encoding genes contains at least one ATTTA sequence in the stretch of untranslated nucleotides at the 3' end.

7. A vector or a corresponding baculovirus according to claims 3, 4, 5 or 6, comprising genes encoding the alpha and beta subunits of bovine follicle stimulating hormone.

8. An insect cell comprising a vector and/or baculovirus according to any one of claims 3-7.

9. A method for producing bovine gonadotropin-like polypeptides or fragments thereof comprising culturing a cell according to claim 8 in a suitable medium and harvesting the polypeptide from the culture.

10. The use of recombinant bovine follicle stimulating hormone -or parts of it- according to claim 2 in super-ovulation treatment, or in the treatment of reproductive problems such as anoestrus and incomplete follicle development.

11. Recombinant bovine follicle stimulating hormone obtainable by a method according to claim 1 at a production level of at least 1 $\mu g/ml^{-1}$ in an ACA.

12. The use of recombinant bovine follicle stimulating hormone -or parts of it- according to claim 2, in the human.

13. The use of recombinant bovine follicle stimulating hormone -or parts of it- according to claim 2 in *in vitro* oocyte-maturation and fertilization.

Table 1.
Comparison of production of recombinant gonadotropic hormone (subunit) according to published data

lit. reference	expression system	matrix	rec. expression product	max.prod $\mu g^{-1} ml^{-1}. 24h^{-1}$	method	ref. prep.	max prod $IU.ml^{-1} 24h^{-1}$	method	ref. prep	remarks
Chappel '88	C127 mouse epitheloid cells		rbFSHαß					G.C./prog Steelman Pohley	USDA -FSH	
Keene '89	CHO cells	α MEM	rhFSHαß	0.6	G.C. aromatase assay	hFSH-LER-907	1.1	G.C. aromatase assay	hFSH-LER-907	
v. Weezenbeek '90	CHO cells	medium	rhFSHαß	84*	Steelman-Pohley	urinary FSH/hMG	650	Steelman-Pohley	urinary FSH/hMG	continuous perfusion system *FSH/hMG: 7778 IU.mg^{-1}
Greenberg '91	transgenic mice	milk	rbFSHαß	2600	RIA	USDA-B5				
				15.3*	RRA	NIH FSH S9	67	RRA	NIH FSH S9	*NIH FSH S9: 4000 IU.mg^{-1}
							66	G.C./E_2	NIH FSH S9	
Chen, Shen & Bahl '91	baculo	Grace medium	rhCGß	1.5	RIA	hCGß		RRA Leydig cell/ cAMP/prog.	hCGαß	
Chen & Bahl '91	baculo	Grace medium	rhCGαß		RIA			RRA Leydig cell/ cAMP/prog.	hCGαß	
Huang '91	baculo	TNM FH medium	r carp GTHα	4.5	RIA	pituitary carp GTHα		carp testis/T	pituitary carp GTHα	
Nakhai, Sridhar, Talwar, Hasnain '91	baculo	medium	rhCGα	11.3	RIA	hCGα		RRA Leydig cell/T	hCGαß	
Nakhai '91	baculo	medium	rhCGα	11.3	RIA	hCGα	2[b]	RRA Leydig cell/T	hCGαß	[b] calculated on hCG:10.000 IU.mg^{-1}
Nakhai '92	baculo	medium	rhCGß	8.02	RIA	hCGß	17[b]	RRA	hCGαß	
							13[b]	Leydig cell/T	hCGαß	
Jha '92	baculo	larva body tissue	rhCGß	1.2*	RIA	hCGß	6[b,d]	Leydig cells/T	hCGαß	* after 96 hrs [d] per larva
		hemo-lymph		1.4*	RIA	hCGß	2[b,d]	Leydig cells/T	hCGαß	
Sridhar '93	baculo	medium	rhCGß	11.3	RIA	hCGß	90[b]	Leydig cell/T RRA	hCGαß hCGαß	
Roth '93	CHO cells	D MEM	rhFSHαß	1.0	RIA	pituary hFSH		RRA	pituary hFSH	

lit. reference	expression system	matrix	rec. expression product	max prod $\mu g^{-1}.ml^{-1}$. $24h^{-1}$	method	ref. prep.	max prod $IU.ml^{-1}$ $24h^{-1}$	method	ref. prep	remarks
Sriller & Ilsenbeck '93	baculo	medium	rhCGß		Western blot	hCGß				
Hassiotou '94	baculo	medium	rhCGß	8.55	RIA	hCGß	18[1]	RIA	hCGß	
							13[1]	Leydig cell/T	hCGß	
Mountjoy '94	CHO cells	α MEM	rofSHß	0.062	RRA	NIDDK-oFSH RP-1	0.02	RRA	NIDDK-oFSH RP-1	NIDDK-oFSH RP-1: 20 $U.mg^{-1}$
							0.03	Sertoli cell/E2		
Dias '94	baculo	TNM FH	rhFSHß	8-10	RIA/ELISA	pituary hFSH		RRA Y_1 cell assay	pituary hFSH	
Lindau-S. '94	baculo	Grace medium	rhFSHß	1-2	ELISA	pituitary FSH		RRA Y_1 cell/ cAMP	pituitary FSH	
V.d. Wiel '94 (this report)	baculo	Sf900 medium	rbFSHß	1-5	ELISA	bFSH-028	20	Y_1 cell/cAMP	USDA-bFSH-I-2	USDA-bFSH-I-2: 854 $IU.mg^{-1}$

Abbreviations are:
r = bovine
r = human
r = ovine

G.C. = granulosa cell
preg. = progesterone
arom. = aromatase

RRA = radio receptor assay
E2 = oestradiol-17ß
T = testosterone

hCG = human chorionic gonadotrophin

Cont. of Table 1

Table 2

Production level[1] (IU/ml for bioassays, and µg/ml for ACA and specific activity[2] (IU/µg) of rbFSH	
\batch \ assay \	1/7/94
Y_1 morphol[3]	8.54 8.54 8.54 4.27
x ± S.D.	7.47 ± 2.14
S.A.	2.49
Y_1 cAMP[4]	19.1 29.9 23.9
x ± S.D	24.3 ± 5.41
S.A	8.1
Sertoli cell[4]	13.7 4.4 2.7
x ± S.D.	6.90 ± 4.83
S.A.	2.3
OMI	15.0 31.0
x ± S.D.	23.0 ± 11.3
S.A.	7.7
ACA	1.8 1.6 5.6
x ± S.D.	3.0 ± 1.8

1) harvest at 72 hours after infection (p.i.), except when indicated
2) S.A. $\frac{\text{IU/ml (bioassay)}}{\text{µg/ml (ACA)}}$

3) measurement of change in cell morphology
4) measurement of cAMP (1/2 max.level), except when indicated.

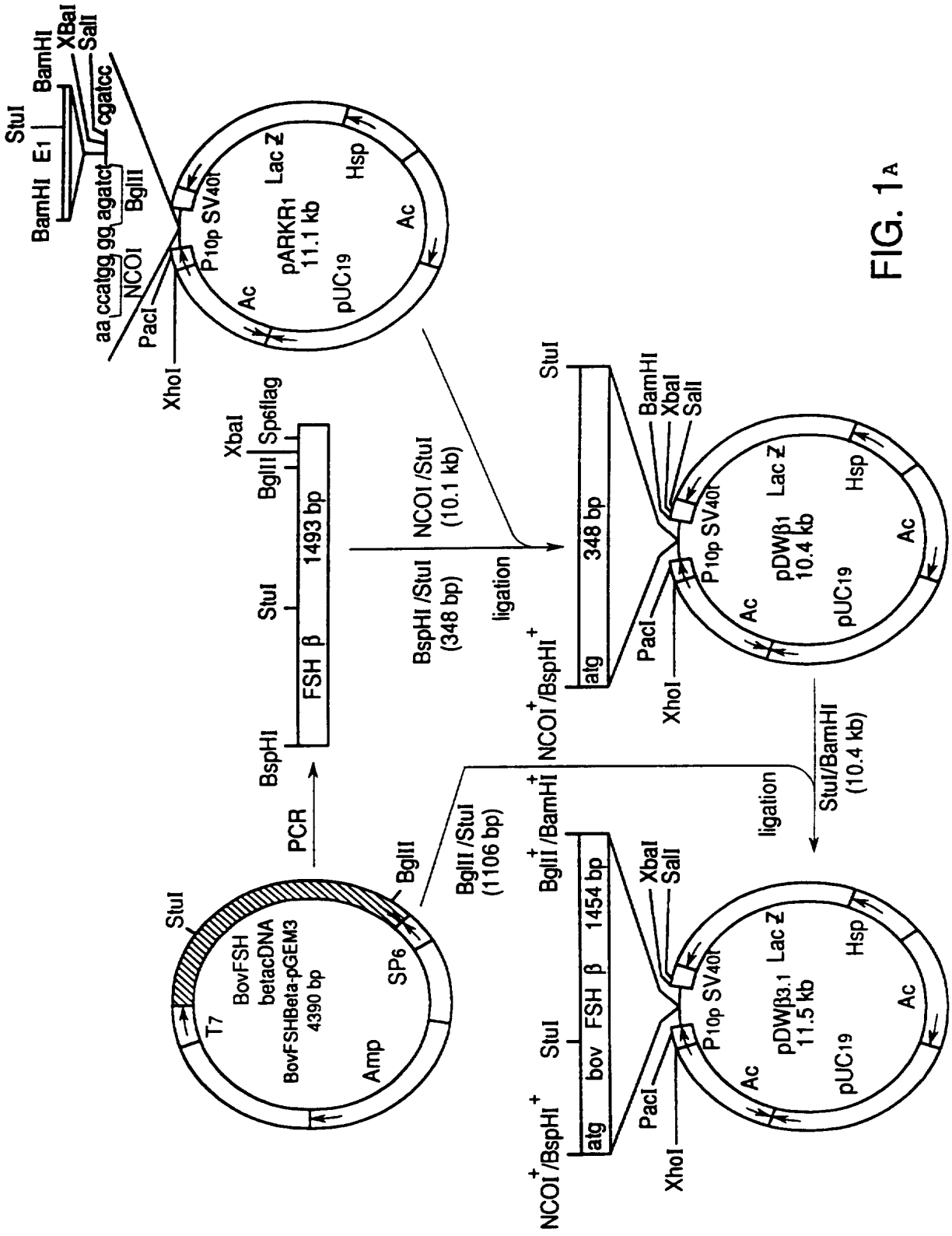
StuI
BamHI
XbaI
SalI
E1
cgatcc
agatct
BglII
aa ccatgg gg
NCOI
PacI
XhoI
P10p SV40t
pARKR1
11.1 kb
pUC19
Lac Z
Hsp
Ac
PCR
BspHI
FSH β
1493 bp
BglII
Sp6flag
BspHI /StuI
(348 bp)
NCOI /StuI
(10.1 kb)
ligation
NCOI+/BspHI+
348 bp
atg
pDWβ1
10.4 kb
BglII /StuI
(1106 bp)
BglII+/BamHI+
StuI/BamHI
(10.4 kb)
NCOI+/BspHI+
1454 bp
bov
pDWβ3.1
11.5 kb
T7
BovFSH
betacDNA
BovFSHBeta-pGEM3
4390 bp
Amp
SP6

FIG. 1A

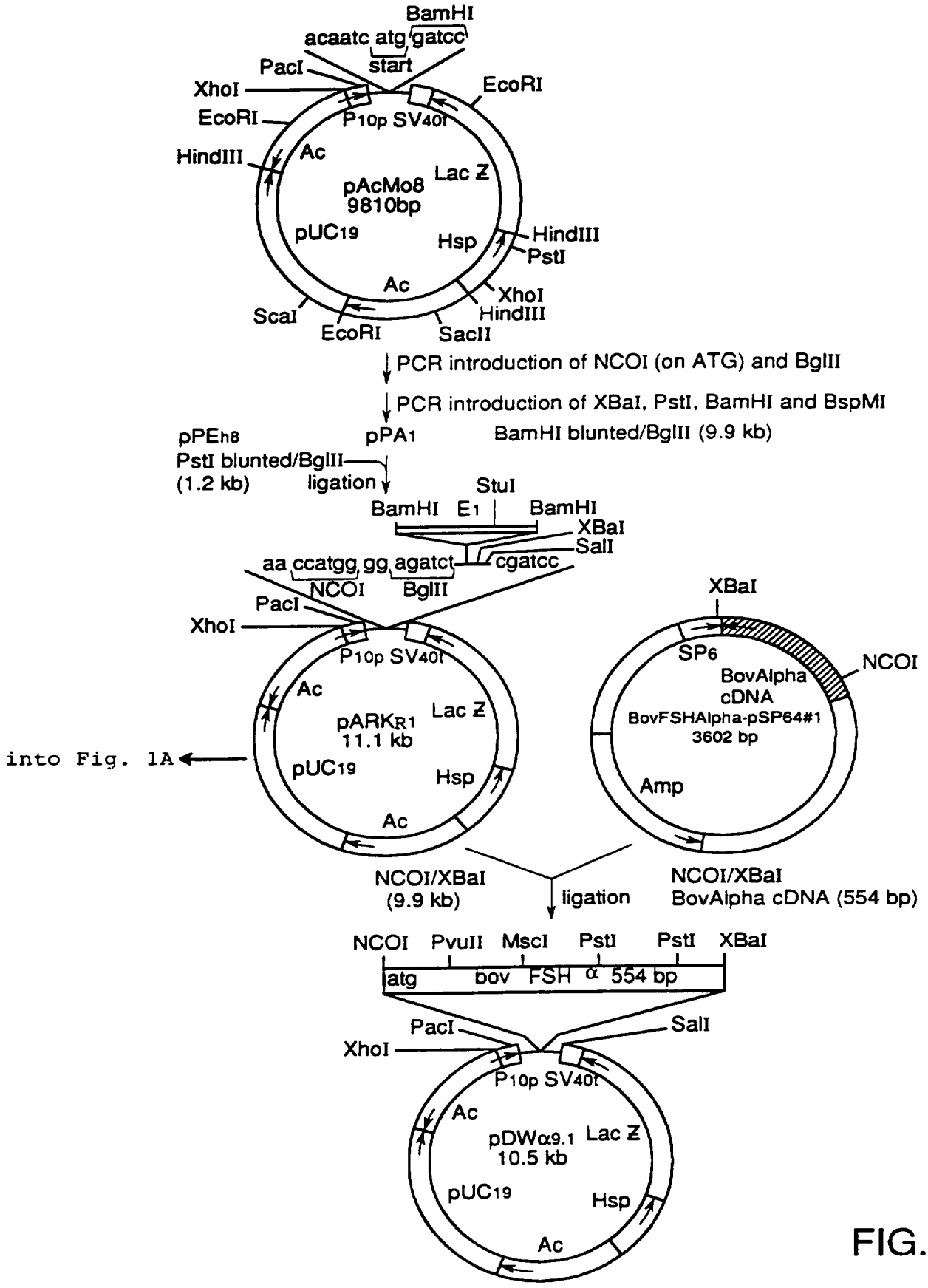
BamHI
acaatc atg gatcc
start
PacI
XhoI
EcoRI
EcoRI
P10p SV40t
HindIII
Ac
Lac Z
pAcMo8
9810bp
pUC19
Hsp
HindIII
PstI
Ac
XhoI
HindIII
ScaI
EcoRI
SacII
PCR introduction of NCOI (on ATG) and BglII
PCR introduction of XBaI, PstI, BamHI and BspMI
pPEh8
PstI blunted/BglII
(1.2 kb)
pPA1
ligation
BamHI blunted/BglII (9.9 kb)
StuI
BamHI E1
BamHI
XBaI
SalI
aa ccatgg gg agatct
cgatcc
NCOI
BglII
PacI
XhoI
P10p SV40t
Ac
Lac Z
pARKR1
11.1 kb
pUC19
Hsp
Ac
into Fig. 1A
XBaI
SP6
NCOI
BovAlpha
cDNA
BovFSHAlpha-pSP64#1
3602 bp
Amp
NCOI/XBaI
(9.9 kb)
ligation
NCOI/XBaI
BovAlpha cDNA (554 bp)
NCOI
PvuII
MscI
PstI
PstI
XBaI
atg
bov
FSH
α
554 bp
PacI
SalI
XhoI
P10p SV40t
Ac
Lac Z
pDWα9.1
10.5 kb
pUC19
Hsp
Ac

FIG. 1B

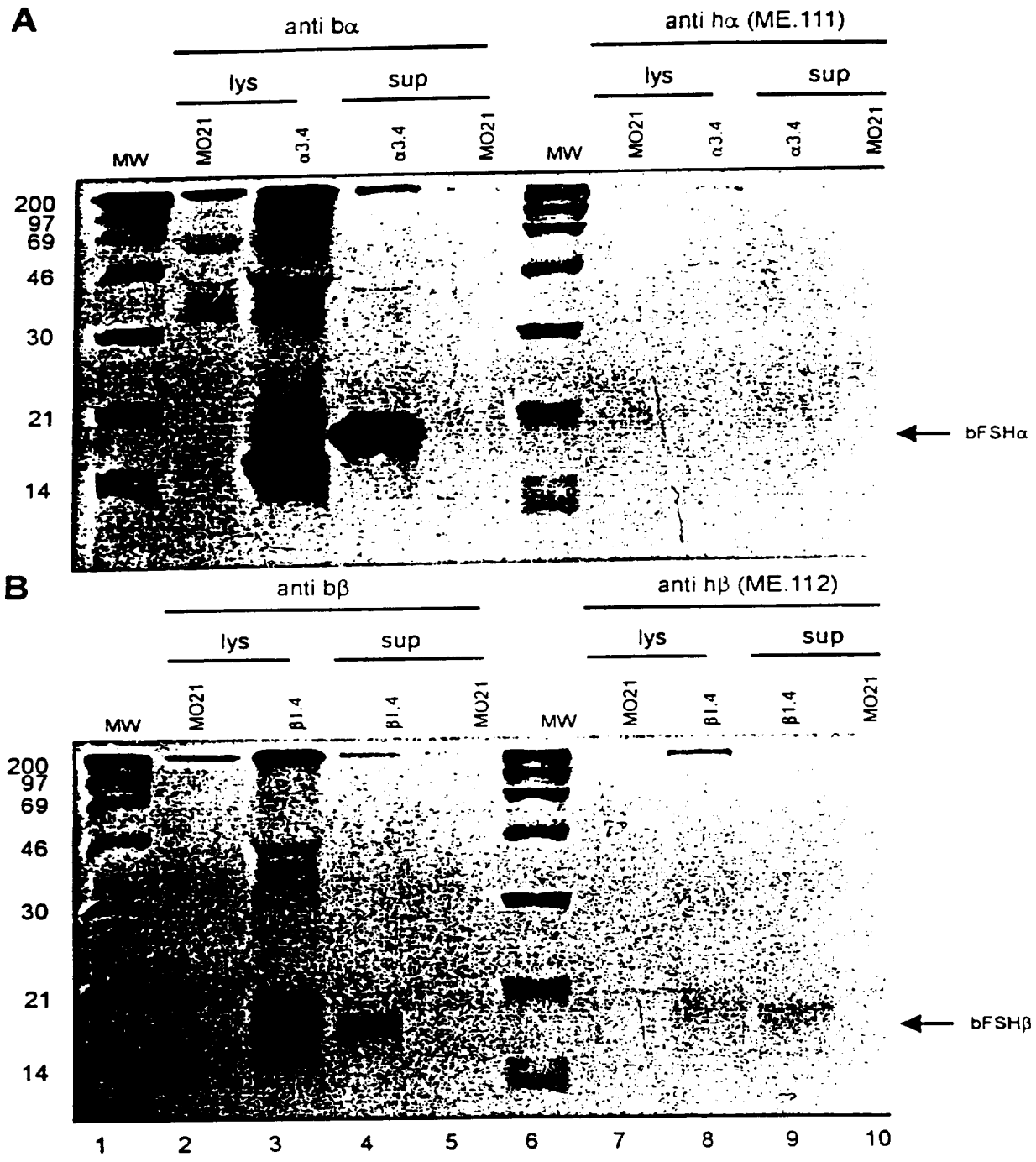
A
anti bα
anti hα (ME.111)
lys
sup
lys
sup
MW
MO21
α3.4
α3.4
MO21
MW
MO21
α3.4
α3.4
MO21
200
97
69
46
30
21
14
bFSHα
B
anti bβ
anti hβ (ME.112)
lys
sup
lys
sup
MW
MO21
β1.4
β1.4
MO21
MW
MO21
β1.4
β1.4
MO21
200
97
69
46
30
21
14
bFSHβ
1
2
3
4
5
6
7
8
9
10

FIG. 2

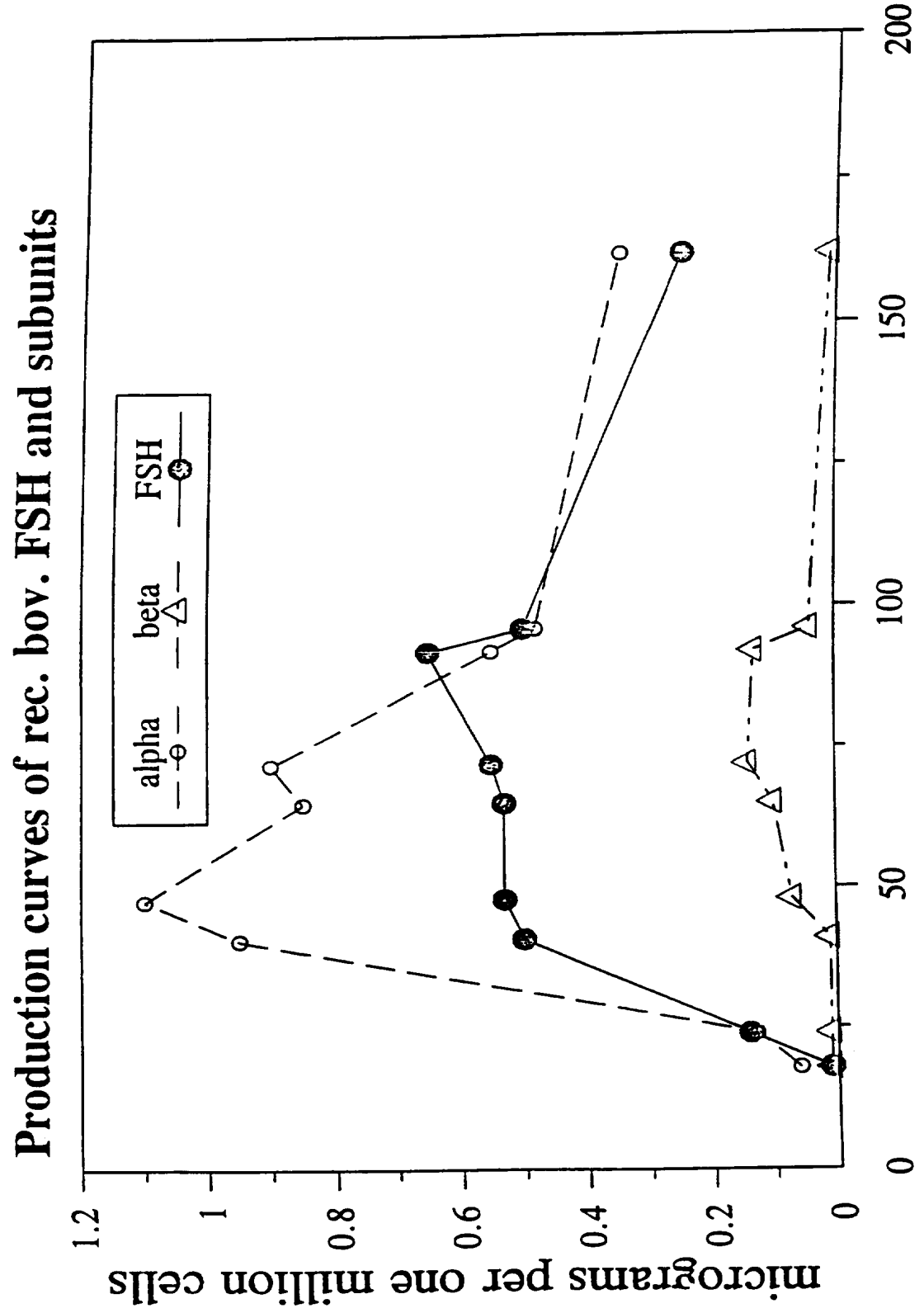
Production curves of rec. bov. FSH and subunits
alpha
beta
FSH
micrograms per one million cells
hours after infection
0
0.2
0.4
0.6
0.8
1
1.2
0
50
100
150
200
bLH alpha: AFP 3111 A
bFSH beta: USDA bFSH beta
bFSH: UCB i 058

FIG. 3

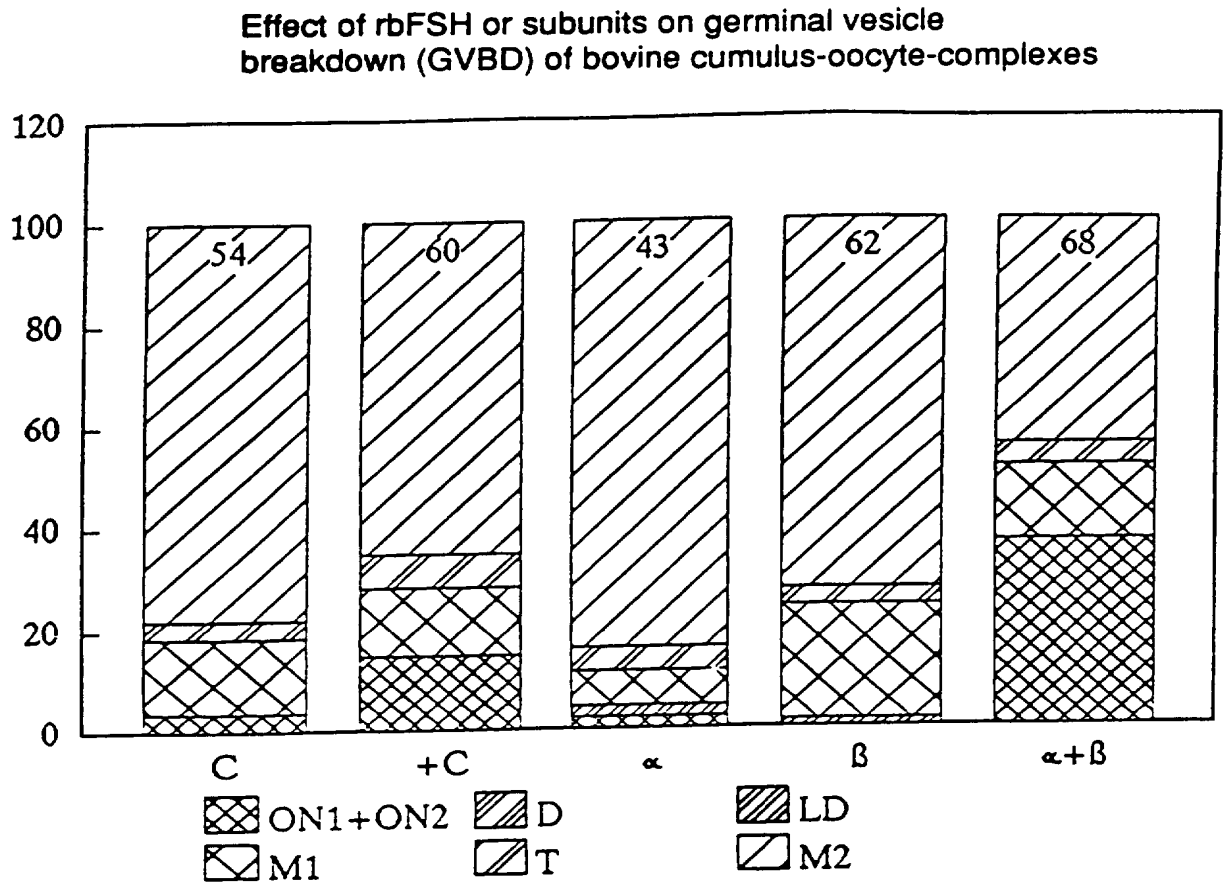
Effect of rbFSH or subunits on germinal vesicle breakdown (GVBD) of bovine cumulus-oocyte-complexes
120
100
80
60
40
20
0
54
60
43
62
68
C
+C
α
ß
α+ß
ON1+ON2
D
LD
M1
T
M2

FIG. 4

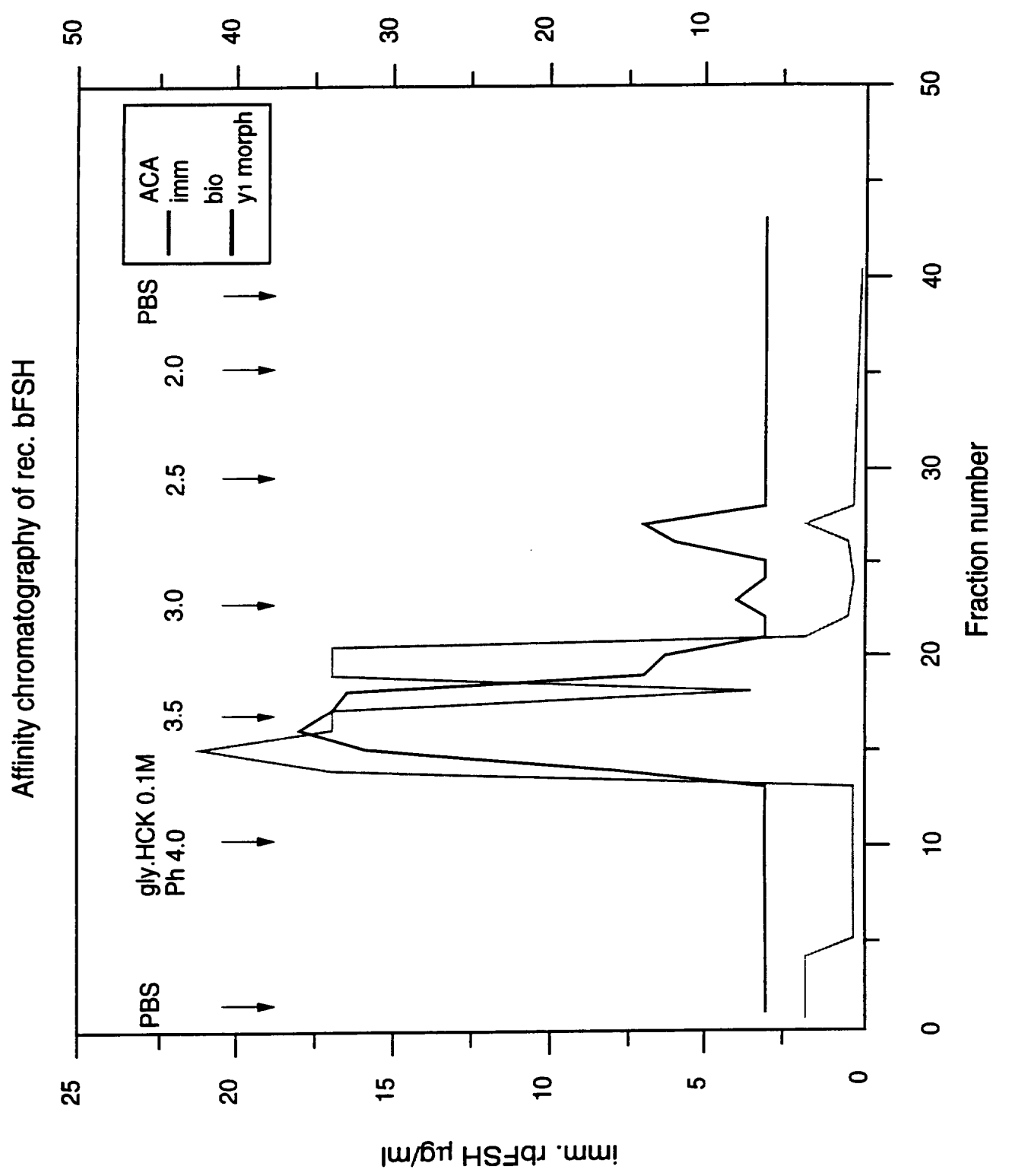
Affinity chromatography of rec. bFSH
imm. rbFSH µg/ml
in vitro bioactivity I.U./ml
Fraction number
PBS
gly.HCK 0.1M
Ph 4.0
3.5
3.0
2.5
2.0
PBS
ACA
imm
bio
y1 morph
0
5
10
15
20
25
10
20
30
40
50

Fig. 5

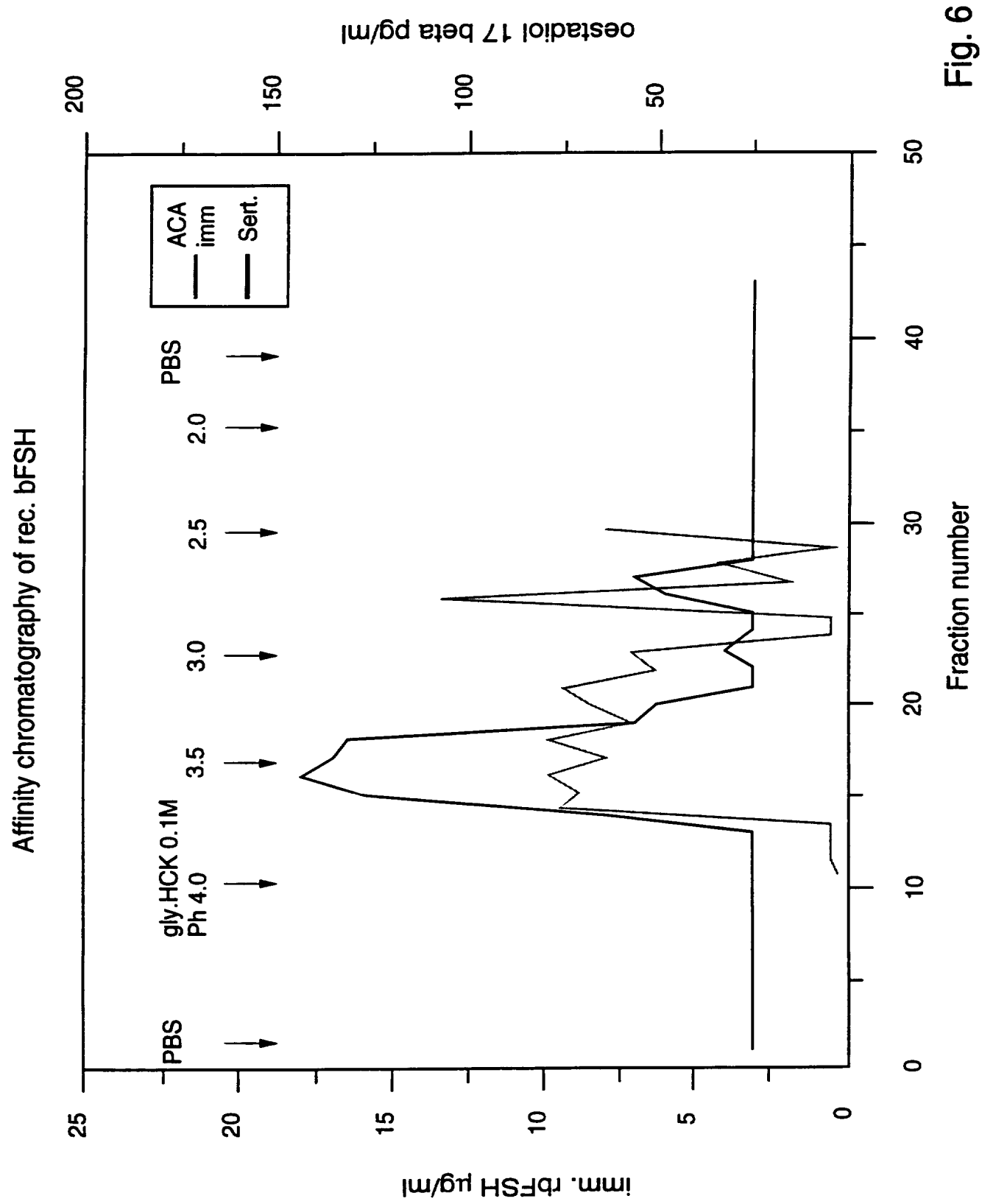
Affinity chromatography of rec. bFSH
imm. rbFSH µg/ml
oestadiol 17 beta pg/ml
Fraction number
ACA imm
Sert.
PBS
gly.HCK 0.1M Ph 4.0
3.5
3.0
2.5
2.0
PBS
0
5
10
15
20
25
50
100
150
200
0
10
20
30
40
50

Fig. 6

INTERNATIONAL SEARCH REPORT

Inv onal Application No
PCT/NL 96/00073

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/16 C12N15/86 C07K14/59 A61K38/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOLECULAR BIOLOGY OF THE CELL, vol. 4, 1993, page 136a XP002003576 SHARMA,S.C. ET AL.: "Expression of bovine alpha and beta follicle stimulating hormone in baculovirus" cited in the application	1,3-9
Y	Thirty-third annual meeting of the american society for cell biology, New Orleans, Louisiana, USA; December 11-15, 1993; see abstract no. 791; ---	11,13
X	EP,A,0 276 166 (DONALDSON LLOYD E) 27 July 1988 see the whole document ---	10
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 May 1996	07.06.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO,A,90 13627 (GRANADA BIOSCIENCES INC) 15 November 1990 see the whole document	13 11
Y	EP,A,0 404 458 (BUNGE AUSTRALIA) 27 December 1990 see the whole document	13
Y	WO,A,90 02757 (INTEGRATED GENETICS INC) 22 March 1990 see the whole document	1-9
Y	DATABASE WPI Section Ch, Week 9423 Derwent Publications Ltd., London, GB; Class B04, AN 94-185924 XP002003581 & JP,A,06 121 687 (NIPPON SEIBUTSU KAGAKU KENKYUSHO ZH) , 6 May 1994 cited in the application *see the whole document* see abstract	1-9
X	CHEMICAL ABSTRACTS, vol. 120, no. 9, 28 February 1994 Columbus, Ohio, US; abstract no. 96092, WILSON, J. M. ET AL: "Superovulation of cattle with a recombinant-DNA bovine follicle stimulating hormone" XP002003911 see abstract & ANIM. REPROD. SCI. (1993), 33(1-4), 71-82 CODEN: ANRSDV;ISSN: 0378-4320, 1993,	2,10-13
X	JOURNAL OF ENDOCRINOLOGY, vol. 137, 1993, pages 59-68, XP002003575 WU, J.-B. ET AL.: "Isolation of FSH from bovine pituitary glands" cited in the application *see the whole application*	2,11
A	BIOCHEMISTRY, vol. 22, 1983, pages 4856-4860, XP002003577 ERWIN, C.R. ET AL.: "Nuecleotide sequence of cloned complementary deoxyribonucleic acid for the alpha subunit of bovine pituitary glycoprotein hormones" cited in the application *see the whole document*	1,3

1

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PC I/NL 96/00073

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 83, 1986, WASHINGTON US, pages 6618-6621, XP002003578 ESCH, F.S. ET AL.: "Cloning and DNA sequence analysis of the cDNA for the precursor of the beta chain of bovine follicle stimulating hormone" cited in the application *see the whole document* ---	1,4
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 41, 1994, MD US, pages 25289-25294, XP002003579 DIAS, J.A. ET AL.: "Receptor binding and functional properties of chimeric human follitropin prepared by an exchange between a small hydrophilic intercysteine loop of human follitropin and human lutropin" cited in the application *see the whole document* -----	1

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NL 96/00073

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0276166	27-07-88	US-A-	4780451	25-10-88
		AU-B-	1025988	28-07-88
		CA-A-	1311413	15-12-92
		US-A-	5162306	10-11-92
WO-A-9013627	15-11-90	AU-B-	5531090	29-11-90
		CA-A-	2015707	01-11-90
EP-A-0404458	27-12-90	NONE		
WO-A-9002757	22-03-90	EP-A-	0394363	31-10-90